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Characterization of the Protective Effects of Melatonin and Related Indoles Against α-Naphthylisothiocyanate-Induced Liver Injury in Rats

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Abstract The protective effect of melatonin, 6-hydroxymelatonin and *N*-acetylserotonin against α-naphthylisothiocyanate (ANIT)-induced liver injury was investigated and compared in rats injected once with the hepatotoxicant (75 mg/kg body weight). In rats injected with ANIT alone, liver injury with cholestasis developed within 24 h, as indicated by both serum levels of alanine aminotransferase (SGPT) and aspartic acid aminotransferase (SGOT) activities and serum total bilirubin concentration. The administration of melatonin or 6-hydroxymelatonin (10 mg/kg body weight) to ANIT-injected rats reduced significantly the serum levels of both SGPT and SGOT and the serum total bilirubin concentration. For all hepatic biochemical markers, melatonin was more effective that 6-hydroxymelatonin. By comparison, the administration of *N*-acetylserotonin (10 mg/kg body weight) to ANIT-injected rats did not reduce the serum levels of either hepatic enzymes or the serum total bilirubin concentration. In ANIT-injected rats, hepatic lipid peroxidation (LPO) was significantly higher than in control animals and this increase was significantly reduced by either melatonin, 6-hydroxymelatonin or *N*-acetylserotonin. Furthermore, ANIT treatment caused a significant reduction in liver microsomal membrane fluidity and this reduction was completely reversed by the three indoles. The liver from ANIT-injected rats showed several histopathological alterations; above all there was an acute infiltration of polymorphonuclear neutrophils and an increase in the number of apparent apoptotic hepatocytes. The concurrent administration of melatonin reduced the severity of all morphological alterations, specially the neutrophil infiltration and the number of presumed apoptotic cells. On the contrary, the administration of 6-hydroxymelatonin or *N*-acetylserotonin did not provide any protective effect in terms of the histopathological alterations. These results indicate that melatonin protects against ANIT-induced liver injury with cholestasis in rats, and suggests that this protective effect is likely due to its antioxidant properties and above all to its capacity to inhibit liver neutrophil infiltration, a critical factor in the pathogenesis of ANIT-induced liver injury. 6-hydroxymelatonin, although able to provide partial protection against the ANIT-induced hepatic injury, probably through its antioxidant properties by mechanisms that are unclear, was unable to reduce neutrophil infiltration. Finally, *N*-acetylserotonin in the experimental conditions of this study, only exhibited some antioxidant protection but had no protective effect against ANIT-induced hepatic damage. J. Cell. Biochem. 80:461–470, 2001. © 2001 Wiley-Liss, Inc.

Key words: melatonin; indoles; α-naphthylisothiocyanate; liver injury; lipid peroxidation; membrane fluidity

α-naphthylisothiocyanate (ANIT) is a well-known toxic substance that produces a cholangiolitic hepatitis characterized by intrahepatic cholestasis, hepatocellular and biliary epithelial cell necrosis, and bile duct obstruction [Plaa and Priestly, 1977; Roth and Dahm, 1997]. An inflammatory response in the periportal regions of liver lobules leads to edema and a pronounced infiltration of polymorphonuclear neutrophils into hepatic tissue occurs before the onset of overt liver damage in rats intoxi-

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cated once with ANIT [Goldfarb et al., 1962]. ANIT has attracted attention because it produces, in certain animal species, hepatic lesions that resemble those occurring in biliary cirrhosis in humans [Plaa and Priestly, 1977]. In addition, cholestasis and hepatic lesions resulting from the administration of certain drugs (e.g., erythromycin estolate, chlorpromazine, and others) to humans are mimicked by ANIT administration to rats, so animal models of ANIT hepatotoxicity may by useful for studying the mechanisms of drug-induced cholestasis [Plaa and Priestly, 1977].

The mechanisms of ANIT-induced liver injury have been proposed but they have not been proven [Roth and Dahm, 1997]. In this context, Calcamuggi et al. [1992] suggested that endotoxemia may play a pathogenic role in ANIT-induced liver injury in rats and other studies demonstrated that glutathione plays an important role in ANIT hepatotoxicity [Jean and Roth, 1995]. On the other hand, it has been clearly demonstrated that the pathogenesis of ANIT hepatotoxicity is neutrophil-dependent. Thus, Dahm et al. [1991] reported that neutrophil depletion protects against liver injury in rats treated once with ANIT, while administration of oxygen radical scavengers, such as superoxide dismutase and catalase, do not protect against this hepatic injury. Thus, these authors suggested that the contribution of neutrophils to the development of ANIT-induced liver injury involves a mechanism independent of the production of oxygen radicals. Ohta et al. [1997] reported, however, that in rats intoxicated once with ANIT, serum lipid peroxide (LPO) level increases with the formation and progression of liver injury and that two different Chinese herbal products, which work as oxygen radical scavengers and have anti-inflammatory actions, prevent the progression of ANIT-induced hepatic injury and inhibit the rise in serum lipid peroxidation products. In addition, recently Kongo et al. [1999] suggested that lipid peroxidation might be associated with ANIT-induced liver injury in rats and that the damage to lipids probably occurs via oxygen radicals derived from neutrophils which infiltrate the liver tissue of ANIT-intoxicated rats.

Melatonin, the main pineal secretory product, is known to function as an efficient antioxidant molecule in vitro and in vivo [Reiter, 1996]. Thus, it has been shown that in animals and tissues exposed to agents which induce lipid peroxidation, melatonin provides substantial protection against this oxidative destruction [Reiter, 1996; Longoni et al., 1998]. Additionally, melatonin exerts a protective effect against acute hepatic injuries induced by endotoxic shock [Sewerynek et al., 1995] and ischemia-reperfusion [Sewerynek et al., 1996] in rats through its antioxidant and its inhibitory effects on neutrophil infiltration into the liver tissue. These findings suggest that melatonin may protect against ANIT-induced liver injury with cholestasis in rats because of its antioxidant action and/or its inhibitory effect on neutrophil infiltration. Similar suggestions have been made recently by Ohta et al. [2000a].

In addition to melatonin, other related indoles such as 6-hydroxymelatonin and *N*-acetylserotonin have antioxidant properties in in vitro studies [Chan and Tang, 1996; Matuszak et al., 1997; Wölfler et al., 1999]. However, few studies have investigated and compared the antioxidant properties of different indoles under in vivo conditions [Hara et al., 1997]. In the current study, we investigate and compare the protective effect of melatonin, 6-hydroxymelatonin and *N*-acetylserotonin against ANIT-induced liver injury in rats.

MATERIALS AND METHODS

Chemicals

ANIT, melatonin, 6-hydroxymelatonin, *N*-acetylserotonin, and bovine serum albumin were purchased from Sigma (St. Louis, MO) and 1-(4-trimethyl-ammoniumphenyl)-6-phenyl-1,3,5-hexatriene-p-toluene-sulfonate (TMA-DPH) was purchased from Molecular Probes (Eugene, OR). The Bioxytech LPO-586 kit, purchased from Cayman Chemical (Ann Harbor, MI), was used for measuring the lipid peroxidation products, malonaldehyde (MDA) and 4-hydroxyalkenals (4-HDA). Apoptosis was morphologically detected in situ by terminal deoxynucleotidyl transferase assay (Apoptag Kit, Oncor, Inc. USA). Total serum bilirubin, serum alanine aminotransferase (SGPT) and serum aspartic acid aminotransferase (SGOT) were assayed using commercial test kits from Bayer Diagnostic. All other chemicals used were of analytical grade and were purchased from commercial sources.

Animals

All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory animals. Male Sprague-Dawley rats, weighing 200–250 g, were purchased from Harlan (Houston, TX) and housed three per plexiglass cage. The animal rooms were windowless with automatic temperature control ($22 \pm 2°C$) and lighting (light on at 0700 hours and off at 2100 hours; 14 h light and 10 h dark). Animals received standard laboratory chow and water ad libitum.

Experimental Procedures

After 1 week of acclimation, the animals were divided in eight groups as follows: group I (control), group II (melatonin), group III (6-hydroxymelatonin), group IV (*N*-acetylserotonin), group V (ANIT), group VI (ANIT+melatonin), group VII (ANIT+6-hydroxymelatonin), and group VIII (ANIT+*N*-acetylserotonin). Prior to the injections, the rats were fasted for 12 h, but had free access to water. For the experiment, groups V–VIII were intraperitoneally injected with ANIT dissolved in olive oil at a dose of 75 mg/kg body weight [Ohta et al., 2000a; Kongo et al., 1999]. The groups I–IV received an intraperitoneal injection of the same volume of olive oil. Thirty minutes before the ANIT injections and at 6, 12, and 18 h after toxin injection, melatonin (groups II and VI), 6-hydroxymelatonin (groups III and VII) or *N*-acetylserotonin (groups IV and VIII) was intraperitoneally administered at a dose of 10 mg/kg body weight. The three indoles were dissolved in ethanol and thereafter diluted in saline (the final concentration of ethanol was 1%). Groups I (control) and V (ANIT) received the dose intraperitoneally and at the same time the same volume of ethanol/saline solution. This scheme of indole administration has been previously used and found effective in reducing oxidative damage [Sewerynek et al., 1995; Carneiro and Reiter, 1998]. Beginning at the time of ANIT administration and continuing to the end of the study all animals had free access to food and water. The animals were sacrificed by decapitation. After decapitation, blood and liver samples were quickly collected. The collected blood was centrifuged to obtained serum. Serum and liver samples were frozen at −80°C until use.

Assay of Serum Enzymes and Total Bilirubin

Serum alanine aminotransferase (SGPT) and the aspartic acid aminotransferase (SGOT) were measured automatically (COBAS INTEGRA, Roche) using a commercially prepared kit (Roche). These enzyme activities are expressed as international units/litre (IU/L). Total serum bilirubin also was measured automatically (COBAS INTEGRA, Roche) using a kit from Roche. The serum SGPT and SGOT were used as indices of hepatic cell damage and the total bilirubin as an index of cholestasis.

Assay of Hepatic LPO and Protein

To analyze for lipid peroxidation, a portion of each liver was homogenized in ice-cold 20 mM Tris buffer, pH 7.4, with a Polytron-like stirrer to produce a 1/10 homogenate. Homogenates were centrifuged at 3,000 rpm for 30 min at 4°C and the supernatant was collected and immediately tested for products of lipid peroxidation. Lipid peroxidation products [malonaldehyde (MDA) and 4-hydroxyalkenals (4-HDA)] were estimated in the supernatants by a colorimetric assay using the Bioxytech LPO-586 kit. In this technique, MDA and 4-HDA react with a chromogenic reagent at 45°C yielding a stable chromophore with maximal absorbance at 586 nm wavelength and provide a convenient index of lipid peroxidation [Esterbauer and Cheeseman, 1990]. The light wavelength and the low temperature of incubation used for the measurements eliminate interference and undesirable artefacts. Results are expressed as nmol MDA+4-HDA mg^{-1} microsomal protein. Liver protein concentrations were measured by the Bradford [1976] method using bovine serum albumin as standard.

Microsome Isolation

The microsomal fraction was isolated as described previously [García et al., 1997]. Briefly, the liver was homogenized 1/10 w/v in 140 mM KCl, 20 mM HEPES buffer, pH 7.4. The suspension was centrifuged at $1{,}000 \times g$ for 10 min and the resulting supernatant was centrifuged at $105{,}000 \times g$ for 60 min. The pellet obtained was re-suspended in the same buffer and centrifuged at $10{,}000 \times g$ for 15 min. Then, the supernatant was re-centrifuged at $105{,}000 \times g$ for 60 min and the final pellet was re-suspended 1/1 v/v in the same buffer and stored at −80°C until assay. After isolation,

microsomal membrane fluidity and MDA+4-HDA concentrations were determined.

Membrane Fluidity

Membrane fluidity was measured using the TMA-DPH as probe as described by García et al. [1997]. Briefly, a suspension of 0.5 mg microsomal protein in 50 mM Tris–HCl buffer, pH 7.4 (3 ml final volume) was vigorously mixed on a vortex with TMA-DPH (66.7 nM) for 1 min and incubated with shaking for 30 min at 37°C to ensure the uniform distribution of the fluorescent probe in the microsomes. Polarization parameters (average of 30 observations for each determination) were carried out in a Perkin-Elmer LS-50 luminiscence spectrometer equipped with a circulatory water bath to maintain the temperature of 22 ± 0.1°C. TMA-DPH was excited at 360 nm and its emission recorded at 430 nm. The degree of polarization (P) was calculated using following equation:

$$P = \frac{I_{V_V} - GI_{V_H}}{I_{V_V} + GI_{V_H}}$$

I_{V_V} and I_{V_H} are the emission intensity of vertically polarized light detected by an analyzer oriented parallel or perpendicular, respectively, to the excitation plane and G is a correction factor for the optical system. Results of membrane fluidity were expressed as the inverse of P [García et al. 1997].

Morphological Analysis

For the histological studies, a small portion of six livers was obtained from rats of each group. These were fixed in 4% paraformaldehyde buffered with phosphate solution (0.1 M, pH 7.4) at room temperature. Liver fragments were washed in phosphate buffer and dehydrated in graded concentrations of ethanol (70, 80, 90 and 100%); the fragments were embedded in Paraplas-Plus. From each liver, 4 μm thick sections were obtained and stained with hematoxylin-eosin to evaluate hepatic morphology. Polymorphonuclear neutrophils were counted in 10 randomly selected high-power fields (×650) using an image analysis system (analySIS 2.0).

Morphological Apoptosis Analysis

Apoptosis is often preceded by internucleosomal DNA fragmentation. Apoptotic cells were morphologically detected in situ by terminal deoxynucleotidyl transferase assay and counted under a light microscope at a magnification of 250 ×; in each field the number of apoptotic cells was assessed. A total of 50 periportal areas per rat liver were randomly examined using an image analysis system (analySIS 2.0) in six rats each group.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA). If the values were significant, the Bonferroni test was used to compare the treated and controls groups. The level of significance was accepted at $P < 0.05$.

RESULTS

Serum Levels of Hepatic Enzymes and Total Bilirrubin

Serum SGPT and SGOT activities were determined as indices of hepatic cell damage. In rats injected with ANIT the serum levels of both SGPT and SGOT activities were significantly higher than those determined in the control group (Fig. 1A,B). The administration of melatonin and 6-hydroxymelatonin to ANIT-injected rats reduced significantly the serum levels of SGPT and SGOT activities (Fig. 1A,B). However, in both cases melatonin was more efficient that 6-hydroxymelatonin in reducing the effect of ANIT. On the other hand, the administration of *N*-acetylserotonin did not lower serum activity levels of either SGPT or SGOT (Fig. 1A,B). The injection of melatonin, 6-hydroxymelatonin and *N*-acetylserotonin alone did not affect the activities of these hepatic enzymes in the serum (Fig. 1A,B).

Total bilirubin concentration, which is an index of cholestasis, in the ANIT-injected rats was significantly higher than values obtained in control rats (Fig. 1C). When rats were treated with melatonin, 6-hydroxymelatonin or *N*-acetylserotonin, respectively, melatonin and 6-hydroxymelatonin, but not the *N*-acetylserotonin, reduced significantly total serum bilirubin (Fig. 1C). Melatonin was again more efficient that 6-hydroxymelatonin in reducing the high levels of total bilirubin obtained in ANIT-injected rats. Treatment of rats with melatonin, 6-hydroxymelatonin or *N*-acetylserotonin alone did not affect the total bilirubin concentrations compared to that of control rats (Fig. 1C).

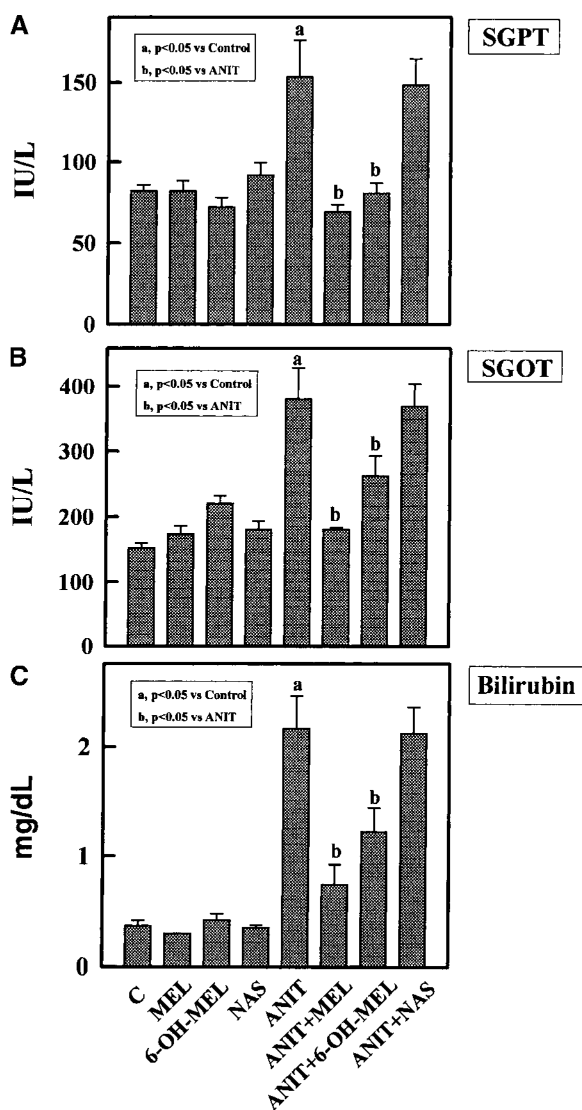

Fig. 1. Effect of intraperitoneal injections of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin (10 mg/kg body weight) on activities of SGPT (**A**), SGOT (**B**), and total bilirubin (**C**) in sera of rats given a single intraperitoneal injection of ANIT (75 mg/kg body weight). Serum SGPT, SGOT, and total bilirubin were assayed in control rats (C), rats injected with melatonin (MEL), 6-hydroxymelatonin (6-OH-MEL), *N*-acetylserotonin (NAS), ANIT (ANIT), ANIT plus melatonin (ANIT+MEL), ANIT plus 6-hydroxymelatonin (ANIT+6-OH-MEL), and ANIT plus *N*-acetylserotonin (ANIT+NAS) as described in Material and Methods. Each value represents the mean ± SEM.

Lipid Peroxidation and Membrane Fluidity

Hepatic lipid peroxidation in the ANIT-injected rats was significantly higher than that in the control group (Fig. 2). When rats were treated with melatonin, 6-hydroxymelatonin or *N*-acetylserotonin, the increased MDA+4-HDA levels induced by ANIT were significantly reduced (Fig. 2). The administration of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin alone did not influence the level of liver lipid peroxidation products (Fig. 2).

As is shown in Figure 3, ANIT caused a significant reduction in microsome membrane fluidity, quantitated as changes in 1/P, with respect to the values of the control group. This reduction in microsome membrane fluidity was completely reversed by melatonin, 6-hydroxymelatonin or *N*-acetylserotonin (Fig. 3). Melatonin, 6-hydroxymelatonin or *N*-acetylserotonin alone did not influence membrane fluidity (Fig. 3).

Morphological Studies

The livers of the control group and the rats injected only with melatonin, 6-hydroxymelatonin or *N*-acetylserotonin exhibited the normal structure (data not shown). Microscopic examination of liver after ANIT administration

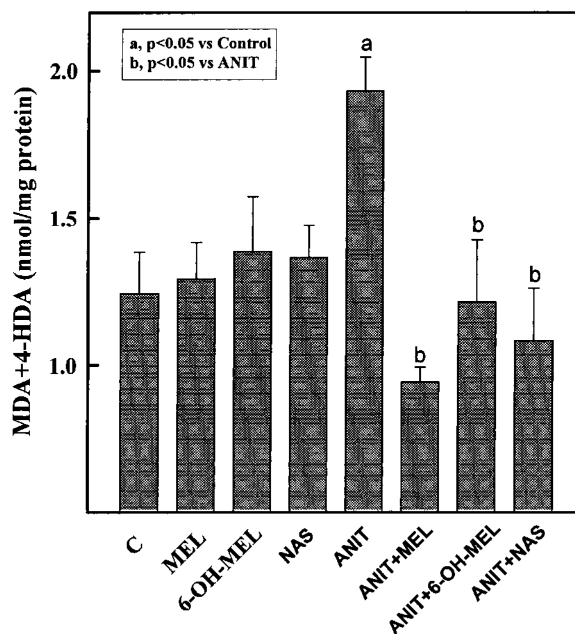

Fig. 2. Effect of intraperitoneal injections of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin (10 mg/kg body weight) on hepatic lipid peroxidation in rats given a single intraperitoneal injection of ANIT (75 mg/kg body weight). Lipid peroxidation is expressed by MDA and 4-HDA concentrations. Hepatic lipid peroxidation was assayed in control rats (C), rats injected with melatonin (MEL), 6-hydroxymelatonin (6-OH-MEL), *N*-acetylserotonin (NAS), ANIT (ANIT), ANIT plus melatonin (ANIT+MEL), ANIT plus 6-hydroxymelatonin (ANIT+6-OH-MEL), and ANIT plus *N*-acetylserotonin (ANIT+NAS) as described in Material and Methods. Each value represents the mean ± SEM.

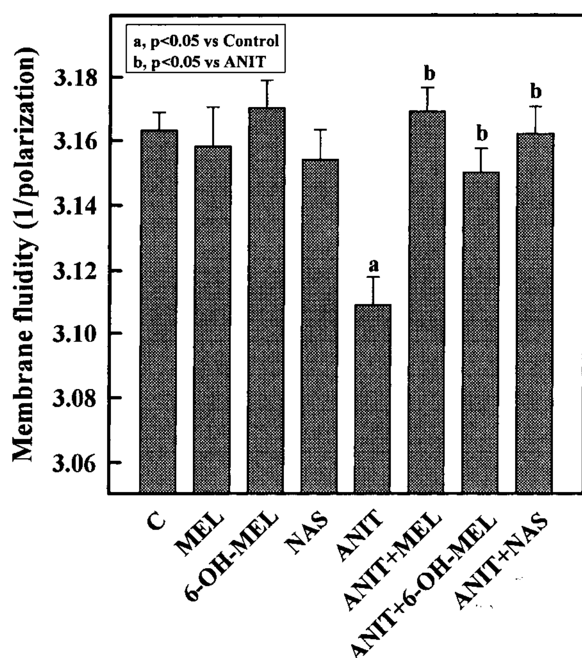

Fig. 3. Effect of intraperitoneal injections of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin (10 mg/kg body weight) on microsomal membrane fluidity (1/P) in rats given a single intraperitoneal injection of ANIT (75 mg/kg body weight). Microsomal membrane fluidity was assayed in control rats (C), rats injected with melatonin (MEL), 6-hydroxymelatonin (6-OH-MEL), *N*-acetylserotonin (NAS), ANIT (ANIT), ANIT plus melatonin (ANIT+MEL), ANIT plus 6-hydroxymelatonin (ANIT+6-OH-MEL), and ANIT plus *N*-acetylserotonin (ANIT+NAS) as described in Material and Methods. Each value represents the mean ± SEM.

showed an acute infiltration with polymorphonuclear neutrophils (Table I). Concurrent melatonin administration reduced the severity of polymorphonuclear neutrophil infiltration (Table I). Injection of 6-hydroxymelatonin or *N*-acetylserotonin did not reduce the polymorphonuclear neutrophil infiltration caused by ANIT (Table I).

Morphological Apoptosis Analysis

The apoptotic cells were found as individual cells in close proximity to the ducts and among the cells comprising the ductual epithelium. Quantification of the number of cells undergoing apoptosis in the periportal area revealed an increase in ANIT-injected rats (Table I). Although apoptotic cells were only quantified in the portal areas, they were detectable in other areas of the liver acinus as well. Concurrent melatonin administration reduced significantly the number of apoptotic cells (Table I). Rats treated with 6-hydroxymelatonin or *N*-acetylserotonin were not protected

TABLE I. Effect of Melatonin and Related Indoles on the Number of Polymorphonuclear Neutrophils (PMNs) and Apoptotic Cells in the Liver After α-naphthylisothiocyanate Administration

Treatment	PMNs	Apoptotic cells
C	10 ± 1.63	6 ± 1.22
MEL	8 ± 1.22	4 ± 0.82
6-OH-MEL	9 ± 2.04	6 ± 0.82
NAS	10 ± 2.04	6 ± 0.82
ANIT	98 ± 8.16	15 ± 1.22
ANIT+MEL	47 ± 5.31	9 ± 0.82
ANIT+6-OH-MEL	87 ± 6.12	16 ± 1.22
ANIT+NAS	96 ± 8.16	15 ± 1.22

The number of polymorphonuclear neutrophils and apoptotic cells were assayed in control rats (C), rats injected with melatonin (MEL), 6-hydroxymelatonin (6-OH-MEL), *N*-acetylserotonin (NAS), ANIT (ANIT), ANIT plus melatonin (ANIT+MEL), ANIT plus 6-hydroxymelatonin (ANIT+6-OH-MEL), and ANIT plus *N*-acetylserotonin (ANIT+NAS). The number of polymorphonuclear neutrophils were assayed in 10 randomly selected high power fields. The number of apoptotic cells were assayed from a total of 50 periportal areas randomly selected per rat liver. Values are expressed as mean ± SEM in six animals in each group.

from ANIT in terms of the number of apoptotic cells (Table I). Control rats and rats treated with only melatonin, 6-hydroxymelatonin or *N*-acetylserotonin exhibited a small number of apoptotic cells (Table I).

DISCUSSION

In the present paper we investigated and compared, under in vivo conditions, the protective effect of melatonin, 6-hydroxymelatonin or *N*-acetylserotonin against the hepatotoxicity of ANIT, a well-characterized hepatotoxin that produces a cholangiolitic hepatitis characterized by intrahepatic cholestasis, necrosis of both hepatocytes and biliary epithelial cells and bile duct obstruction [Plaa and Priestly, 1977; Roth and Dahm, 1997]. Rats injected once with ANIT (75 mg/kg body weight) exhibited liver injury and cholestasis 24 h after the injection, as indicated by the serum activity levels of SGPT and SGOT and total serum bilirubin concentration; this is consistent with previous reports [Kongo et al., 1999; Ohta et al., 2000a]. In our experimental model, melatonin, 6-hydroxymelatonin or *N*-acetylserotonin was intraperitoneally administered to the rats at a dose of 10 mg/kg body weight 30 min before the ANIT injection and 6, 12, and 18 h after ANIT

injection. This experimental injection schedule for melatonin and related indoles has been used in other experimental models and been found effective in reducing oxidative damage [Sewerynek et al., 1995; Carneiro and Reiter, 1998]. Melatonin, and to a lesser degree 6-hydroxymelatonin, as able to protect against ANIT-induced liver injury with cholestasis as demonstrated by the serum values of both SGPT and SGOT activities and total bilirubin concentration. However, *N*-acetylserotonin was unable to protect against the hepatotoxic effects of ANIT. This is the first study in which 6-hydroxymelatonin and *N*-acetylserotonin have been tested as to their potential protective effects against ANIT toxicity.

Previous reports [Kongo et al., 1999; Ohta et al., 2000a] have shown that in rats given a single injection of ANIT, an increase in hepatic LPO levels occurs before discernible morphological injury which is enhanced with the progression of hepatic damage. Accordingly, they have proposed that lipid peroxidation is a significant feature of ANIT-induced hepatic injury [Kongo et al., 1999; Ohta et al., 2000a]. In the present study, hepatic LPO products were consistent with morphological evidence of damage. When rats were treated with either melatonin, 6-hydroxymelatonin or *N*-acetylserotonin, interestingly, the increased hepatic lipid peroxidation products induced by ANIT were reduced in a similar manner by all three indoles.

With respect to melatonin, it is well known that this molecule has antioxidant properties under both in vitro and in vivo conditions [Reiter, 1996; Longoni et al., 1998]. Furthermore, melatonin has been shown to protect against hepatic injury induced by endotoxic and non-endotoxic shock [Sewerynek et al., 1995; El-Sokkary et al., 1999a], ischemia-reperfusion [Sewerynek et al., 1996], ethanol administration [El-Sokkary et al., 1999b], carbon tetrachloride injection [Ohta et al., 2000b], and ligature of extra-hepatic biliary duct [Montilla et al., 2000]. In all these situations, lipid peroxidation induced by oxygen radicals is involved and the protective role of melatonin is believed to result from its antioxidant properties. Consequently, we speculate that the reduction of lipid peroxidation seen in this study, at least in part, was a consequence of the antioxidant properties of melatonin. Moreover, very recently our group has reported the existence of high levels of melatonin in the bile of rats and other mammals [Tan et al., 1999] and we speculate that these high levels of melatonin in the bile may be involved in the protective antioxidant effects of melatonin observed in this study.

With respect to 6-hydroxymelatonin and *N*-acetylserotonin, previous studies have demonstrated that these indoles also have antioxidant properties in several in vitro conditions [Chan and Tang, 1996; Matuszak et al., 1997]. However, few studies have been carried out to investigate the antioxidant properties of these indoles under in vivo conditions [Hara et al., 1997]. Furthermore, in the in vitro studies the results obtained with different indoles have been variable [Wölfler et al., 1999]. In our study, the results obtained with liver LPO were supported by the studies on membrane fluidity. Thus, studies in a variety of membrane systems have demonstrated that free radicals disturb the order and lipid dynamics of the membrane and the biophysical measurements of membrane fluidity are a good method to document membrane structural alterations as a consequence of lipid peroxidation [Curtis et al., 1984]. In this context, two basic mechanisms have been proposed to explain loss in membrane fluidity due to lipid peroxidation. Firstly, the reduction in the unsaturation/saturation ratio of membrane fatty acids [Curtis et al., 1984] and, secondly, the formation of cross-linking of lipid–lipid and lipid–protein moieties [Eichenberger et al., 1982]. Hence, antioxidant molecules that inhibit lipid peroxidation also prevent membrane rigidity induced by oxidative stress. The results obtained in this study, using TMA-DPH as a fluorescence probe, are consistent with these observations. Thus, ANIT caused a significant reduction in hepatic microsome membrane fluidity and this reduction was completely prevented by melatonin, 6-hydroxymelatonin or *N*-acetylserotonin. With respect to melatonin, similar results have been reported from several in vitro studies which used other methods to induce LPO [García et al., 1997, 1998]. However, this is the first study that compared the effects of the melatonin, 6-hydroxymelatonin, and *N*-acetylserotonin on hepatic microsome membrane fluidity under in vivo condition.

In the present study the liver injury induced by ANIT, as indicated by the serum values of SGPT and SGOT activities and total bilirubin concentration, was efficiently reversed by mel-

atonin and to a lesser degree by 6-hydroxymelatonin, but not by *N*-acetylserotonin. On the other hand, membrane oxidative injury induced by ANIT, as shown by both LPO and membrane fluidity was prevented to a similar degree by all three indoles. To explain these differences the pathogenic mechanisms of hepatic injury due to ANIT are summarized. Several mechanisms for the pathogenesis of ANIT-induced liver injury have been proposed; these include endotoxemia [Calcamuggi et al., 1992], the formation of a reversible S-conjugate between the glutathione and the ANIT [Jean and Roth, 1995], and the infiltration of polymorphonuclear neutrophils into the hepatic parenchyma all of which induce hepatic damage. Although glutathione may play a role in ANIT hepatotoxicity, neutrophil infiltration is critical to hepatic pathogenesis caused by ANIT. Thus, in rats given one injection of ANIT, it has been shown by histological studies that polymorphonuclear neutrophils prominently infiltrate the hepatic tissue before the onset of liver injury [Goldfarb et al., 1962]. In this context, Dahm et al. [1991] reported that in rats pretreated with antineutrophil serum, the development of ANIT-induced liver damage was prevented. Because of this they suggested that neutrophil infiltration is a major aspect of ANIT-induced liver injury. Also, it has been demonstrated under in vitro conditions that ANIT stimulates neutrophils to release superoxide anions and proteolytic enzymes that are toxic to hepatic parenchyma cells [Roth and Hewett, 1990; Hill and Roth, 1998]. However, Dahm et al. [1991] found that in rats pretreated with a combination of the antioxidative superoxide dismutase and catalase, the degree of ANIT-induced hepatic damage is not attenuated. They, therefore, suggested that neutrophil-derived oxygen radicals are not involved in the development of ANIT-induced liver injury. Recent reports [Kongo et al., 1999; Ohta et al., 2000a] indicate otherwise. These workers believe that hepatic lipid peroxidation associated with ANIT-induced liver injury involves oxygen radicals derived from neutrophils which infiltrate the liver. Neutrophils are known to mediate lipid peroxidation through the production of superoxide anion via activated NADPH oxidoreductase [Casini et al., 1997]. In the present study histological analyses of liver tissue after ANIT administration showed the infiltration of neutrophils. Furthermore, in the ANIT-injected rats the number of apoptotic hepatocytes was also increased. Concurrent melatonin administration reduced the polymorphonuclear neutrophil infiltration as well as the number of apoptotic hepatocytes. With respect to melatonin, these results are in good accord with previous reports showing that melatonin protects against acute liver injuries induced in rats by endotoxic shock and ischemia-reperfusion, not only through its direct antioxidant action but also by inhibiting the infiltration of polymorphonuclear neutrophils [Sewerynek et al., 1995, 1996]. Collectively these findings indicate that melatonin exerts a protective effect against ANIT-induced liver injury through not only its antioxidant properties, but also because of its inhibitory effect on neutrophil infiltration into the liver tissue.

In contrast to melatonin, the administration of 6-hydroxymelatonin or *N*-acetylserotonin did not protect against neutrophil infiltration and hepatocyte apoptosis. With respect to 6-hydroxymelatonin, its partially protective actions must have been due to actions other than a reduction of neutrophil infiltration. With regard to *N*-acetylserotonin, the current results are in good accordance with previous published findings. Thus, its protective effects appear minimal. However, another possibility is that the results observed in this study with *N*-acetylserotonin may be ascribed to melatonin produced after the administration of *N*-acetylserotonin. It has been proposed that *N*-acetylserotonin stimulates pineal melatonin biosynthesis after its administration [Oxenkrug and Requintina, 1994]. Consequently, the in vivo effects of *N*-acetylserotonin might be better studied in pinealectomized animals or in strains of animals with a defective melatonin biosynthesis system.

The acute hepatotoxicity induced by ANIT in rats is manifested, as mentioned above, as neutrophil-dependent necrosis of not only hepatocellular cells, but also of bile epithelial cells (BECs). In fact, BECs are the primary targets of ANIT-induced toxicity and injury to these cells occurs prior to hepatocellular damage [Connolly et al., 1988]. Furthermore, it has been demonstrated that after exposure to ANIT, BECs produce a factor(s) that causes neutrophil chemotaxis and neutrophil-dependent hepatocellular injury [Hill et al., 1999]. A recent study has demonstrated that human BECs exposed to certain proinflammatory

cytokines (IL-1 and TNFα) rapidly express IL-8 and monocyte chemotactic protein-1 (MCP-1), potent chemotactic agents for neutrophils and monocytes or T cells, respectively [Morland et al., 1997].This is not unusual, since epithelial cells of various types are known to produce chemotactic factors. For example, after exposure to certain toxicants, pulmonary airway epithelial cells and renal epithelial cells produce several chemokines (e.g., macrophage inflammatory protein [MIP], cytokine-induced neutrophil chemoattractant [CINC], and MIP-1) capable of inducing neutrophil chemotaxis and activation [Driscoll et al., 1993; Schmouder et al., 1993]. BECs have active inmunological roles in both innate and adaptive immune responses. In fact, it has been demonstrated that BECs are able to secrete chemokines and cytokines, to express cell adhesion molecules, and to carry out functions as professional antigen-presenting cells [Reynoso-Paz et al., 1999].

In conclusion, from the observations summarized herein and from the results of the present study, we speculate that melatonin protects against ANIT-induced liver injury because of its antioxidant properties and additionally due to its capacity to inhibit hepatic neutrophil infiltration, probably by inhibiting the production in BECs due to one or more factors (probably chemokines) that are responsible for neutrophil chemotaxis. It is important to note that neutrophil infiltration is the first event in inflammation and melatonin has been postulated as an anti-inflammatory agent [Cuzzocrea et al., 1997]. Furthermore, it has been demonstrated that melatonin is able to modulate the production of several cytokines [García-Mauriño et al., 1997]. However, the exact nature of this factor(s) requires further investigations. With respect to 6-hydroxymelatonin, although this indole is able to provide partial protection against ANIT-induced liver injury, probably through its antioxidant properties, its mechanism of action in unclear. Finally, *N*-acetylserotonin, in the experimental conditions of this study, showed less pervasive effects, only reducing ANIT-induced hepatic LPO and the effects on microsomal membrane fluidity.

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REFERENCES

Bradford M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–253.

Calcamuggi G, Lanzio M, Dughera L, Babini G, Emanuelli G. 1992. Endotoxin tolerance and polymyxin B modify liver damage and cholestasis induced by a single dose of α-naphthylisothiocyanate in the rat. Arch Toxicol 66:126–130.

Carneiro RCG, Reiter RJ. 1998. Melatonin protects against lipid peroxidation induced by δ-aminolevulinic acid in rat cerebellum, cortex and hippocampus. Neuroscience 82: 293–299.

Casini A, Ceni E, Salzano R, Biondi P, Parola M, Galli A, Foschi M, Caligiuri A, Pinzani M, Surrenti C. 1997. Neutrophil-derived superoxide anion induces lipid peroxidation and stimulates collagen synthesis in human hepatic stellate cells: role of nitric oxide. Hepatology 25:361–367.

Connolly AK, Price SC, Connolly JC, Hinton RH. 1988. Early changes in bile duct lining cells and hepatocytes in rats treated with alpha-naphthylisothiocyanate. Toxicol Appl Pharmacol 93:208–219.

Curtis MT, Gilfor D, Farber JL. 1984. Lipid peroxidation increases the molecular order of microsomal membranes. Arch Biochem Biophys 235:644–649.

Cuzzocrea S, Zingarelli B, Gilad E, Hake P, Salzman AL, Szabó C. 1997. Protective effect of melatonin in carrageenan-induced models of local inflammation: relationship to its inhibitory effect on nitric oxide production and its peroxynitrite scavenging activity. J Pineal Res 23:106–116.

Chan TY, Tang PL. 1996. Characterization of the antioxidant effects of melatonin and related indoleamines in vitro. J Pineal Res 20:187–191.

Dahm LJ, Schultze AE, Roth RA. 1991. An antibody of neutrophils attenuates α-naphthylisothiocyanate-induced liver injury. J Pharmacol Exp Ther 256:412–420.

Driscoll KE, Simpson L, Carter J, Hassenbein DG, Leikauf, GD. 1993. Ozone inhalation stimulates expression of a neutrophil chemotactic protein, macrophage inflammatory protein-2. Toxicol Appl Pharmacol 119:306–309.

Eichenberger K, Böhni P, Winterhalter KH, Kawato S, Richter C. 1982. Microsomal lipid peroxidation causes an increase in the order of the membrane lipid domain. FEBS Lett 408:297–300.

El-Sokkary GH, Reiter RJ, Cuzzocrea S, Caputi AP, Hassanein A-BMM, Tan DX. 1999a. Role of melatonin in reduction of lipid peroxidation and peroxynitrite formation in non-septic shock induced by zymosan. Shock 12:402–408.

El-Sokkary GH, Reiter RJ, Tan DX, Kim SJ, Cabrera J. 1999b. Inhibitory effect of melatonin on products of lipid peroxidation resulting from chronic ethanol administration. Alcohol 34:842–850.

Esterbauer H, Cheeseman KH. 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Meth Enzymol 186:407–421.

García JJ, Reiter RJ, Guerrero JM, Escames G, Yu BP, Oh ChS, Muñoz-Hoyos A.1997. Melatonin prevents changes in microsomal membrane fluidity during induced lipid peroxidation. FEBS Lett 408:297–300.

García JJ, Reiter RJ, Ortiz GG, Oh CS, Tang L, Yu BP, Escames G. 1998. Melatonin enhances tamoxifen's ability to prevent the reduction in microsomal membrane fluidity induced by lipid peroxidation. J Membrane Biol 162:59–65.

García-Mauriño S, González-Haba MG, Calvo JR, Rafii-El-Idrissi M, Sánchez-Margalet V, Goberna R, Guerrero JM. 1997. Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating $CD4^+$ cells: a possible nuclear receptor-mediated mechanism involving T helper type 1 lymphocytes and monocytes. J Immunol 159:574–581.

Goldfarb S, Singer EJ, Popper H. 1962. Experimental cholangitis due to α-naphthylisothiocyanate (ANIT). Am J Pathol 40:685–698.

Hara M, Iigo M, Ohtani-Kaneko R, Nakamura N, Suzuki T, Reiter RJ, Hirata K. 1997. Administration of melatonin and related indoles prevents exercise-induced cellular oxidative changes in rats. Biol Signals 6:90–100.

Hill DA, Roth RA. 1998. α-naphthylisothiocyanate causes neutrophils to release factors that are cytotoxic to hepatocytes. Toxicol Appl Pharmacol 148:169–175.

Hill DA, Jean PA, Roth RA. 1999. Bile duct epithelial cells exposed to alpha-naphthylisothiocyanate produce a factor that causes neutrophil-dependent hepatocellular injury in vitro. Toxicol Sci 47:118–125.

Jean PA, Roth RA. 1995. Naphthylisothiocyanate disposition in bile and its relationship to liver glutathione and toxicity. Biochem Pharmacol 50:1469–1474.

Kongo M, Ohta Y, Nishida K, Sasaki E, Harada N, Ishiguro I. 1999. An association between lipid peroxidation and α-naphthylisothiocyanate-induced liver injury in rats. Toxicol Lett 105:103–110.

Longoni B, Salgo MG, Pryor WA, Marchiafava PL. 1998. Effects of melatonin on lipid peroxidation induced by oxygen radicals. Life Sci 62:853–859.

Matuszak Z, Reszka, K, Chignell CF. 1997. Reaction of melatonin and related indoles with hydroxyl radicals: EPR and spin trapping investigations. Free Radic Biol Med 23:367–372.

Montilla P, Tunez I, Muñoz MC, Cabrera E, Montilla MC, Plascencia J, De La Torre EJ. 2000. Protective effect of melatonin against oxidative stress induced by ligature of extra-hepatic biliary duct in rats: comparison with the effect of S-adenosyl-L-methionine. J Pineal Res 28:143–149.

Morland CM, Fear J, McNab G, Joplin R, Adams DH. 1997. Promotion of leukocyte transendothelial cell migration by chemokines derived from human biliary epithelial cells in vitro. Proc Assoc Am Physicians 109:372–382.

Ohta Y, Kongo M, Nishida K, Sasaki E, Ishiguro I. 1997. Preventive effects of Saiko-keishi-to and Shigyaku-san extracts on the progression of hepatobiliary injury induced by α-naphthylisothiocyanate. J Trad Med (Wakan Iyakugaku Zasshi) 14:143–148.

Ohta Y, Kongo M, Sasaki E, Ishiguro I, Harada N. 2000a. Protective effect of melatonin against α-naphthylisothiocyanate-induced liver injury in rats. J Pineal Res 29:15–23.

Ohta Y, Kongo M, Sasaki E, Nishida K, Ishiguro I. 2000b. Therapeutic effect of melatonin on carbon tetrachloride-induced acute liver injury in rats. J Pineal Res 28:119–126.

Oxenkrug GF, Requintina PJ. 1994. Stimulation of rat pineal melatonin biosynthesis by *N*-acetylserotonin. Int J Neurosci 77:237–241.

Plaa GL, Priestly BG. 1977. Intrahepatic cholestasis induced by drugs and chemicals. Pharmacol Rev 28: 207–273.

Reiter RJ. 1996. The indoleamine melatonin as a free radical scavenger, electron donor, and antioxidant. In vitro and in vivo studies. Adv Exp Med Bio 398:307–313.

Reynoso-Paz S, Coppel RL, Mackay IR, Bass NM, Ansar AA, Gershwin ME. 1999. The immunobiology of bile and biliary epithelium. Hepatology 30:351–357.

Roth RA, Dahm LJ. 1997. Neutrophil- and glutathione-mediated hepatotoxicity of α-naphthylisothiocyanate. Drug Metab Rev 29:153–165.

Roth RA, Hewett JA. 1990. The cholestatic agent, α-naphthylisothiocyanate, stimulates superoxide release by rat neutrophils in vitro. Lab Invest 62:736–741.

Schmouder RL, Strieter RM, Kunkel SL. 1993. Interferon-gamma regulation of human renal cortical epithelial cell-derived monocyte chemotactic peptide-1. Kidney Int 44:43–49.

Sewerynek E, Melchiorri DA, Reiter RJ, Ortiz GG, Lewinski A. 1995. Lipopolysaccharide-induced hepatotoxicity is inhibited by the antioxidant melatonin. Eur J Pharmacol 283:327–344.

Sewerynek E, Reiter RJ, Melchiorri DA, Ortiz GG, Lewinski A.1996. Oxidative damage in the liver induced by ischemia-reperfusion: protection by melatonin. Hepatogastroenterology 43:898–905.

Tan D-X, Manchester LC, Reiter RJ, Qi W, Hanes MA, Farley NJ. 1999. High physiological levels of melatonin in the bile of mammals. Life Sci 65:2523–2529.

Wölfler A, Abuja PM, Schauenstein K, Liebmann PM. 1999. N-acetylserotonin is a better extra- and intracellular antioxidant than melatonin. FEBS Lett 449:206–210.